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Original article

Fibronectin biosynthesis: influence on fibroblast adhesion during chick embryo development

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Summary — Eight d (8d) and 16d (16d) chick embryo fibroblasts (CEF) exhibited marked differences in their adhesive capacity on plastic support, but not on fibronectin substratum. This suggests differences in fibronectin (FN) expression and/or FN receptor expression. Both 8d and 16d CEF expressed an identical number of membrane receptors for FN with similar affinity. In contrast, the newly synthesized FN appeared *de novo* in 30 min in 8d CEF *versus* 60 min in 16d CEF. This difference is not due to a modification of the polypeptide chain biosynthetic rate. The FN synthesized in 8d CEF became unsensitive to endo β-N-acetyl-glucosaminidase H (endo H) treatment after 20 min, whereas it remained sensitive to endo H until 60 min in 16d CEF. Post-translational modifications of N-linked mannose-rich chains to complex type chain may account for the difference in the expression of cell surface FN and thus for the difference in cell adhesion capacity to plastic.

fibronectin / biosynthesis / adhesion / fibroblast

Résumé — Biosynthèse de la fibronectine et capacités adhésives des fibroblastes d'embryons de poulet. *Les capacités différentes d'adhésion sur le plastique de fibroblastes prélevés à 2 stades du développement embryonnaire chez le poulet (8 et 16 j) sont abolies quand le support est recouvert de fibronectine (FN). Ces résultats suggèrent des différences dans l'expression de la FN et/ou des récepteurs membranaires de la FN au cours du processus d'adhésion. Cependant, les 2 populations de fibroblastes expriment le même nombre de récepteurs d'affinité identique. Par contre, l'apparition de la FN à la surface des cellules est plus rapide à 8j (30 min) qu'à 16 j (60 min). Ce retard observé dans les fibroblastes de 16 j n'est pas dû à une vitesse de synthèse plus lente de la chaîne polypeptidique, mais plutôt à une modification post-traductionnelle de la FN. En effet, l'utilisation de l'endo β-N-acétylglucosaminidase H (endo H) montre que les chaînes oligosaccharidiques de la FN néosynthétisée par les fibroblastes de 16j sont converties en structure complexe beaucoup plus tardivement que celles de la FN des fibroblastes de 8 j. Ces résultats suggèrent un ralentissement du trafic intracellulaire de la FN à 16 j qui peut influer sur la vitesse d'apparition de la FN à la surface cellulaire et sur les capacités adhésives des cellules lors des premières étapes de l'attachement et de l'étalement cellulaire.*

fibronectine / biosynthèse / adhésion / fibroblaste

INTRODUCTION

Fibronectins constitute a group of highly conserved glycoproteins found in plasma and extracellular matrix. They promote cell attachment and spreading (Yamada, 1983) and many important aspects of embryonic development, including cell growth, migration and differentiation are dependent upon cell-extracellular matrix interactions (Hay, 1982). Recent studies have provided evidence for a dynamic distribution and expression of both fibronectin and FN-receptor during the adhesion process, where fibronectin and its receptor co-localize in stationary fibroblasts (Chen *et al,* 1986; Roman *et al,* 1989). Chick embryo fibroblasts exhibit differences in their adhesive capacities on uncoated substratum depending on the stage of development; they behave identically on fibronectin–coated substratum. This could be due to differences in the expression of either the fibronectin receptor or membrane-bound fibronectin. Differences in the expression of membrane fibronectin have been reported in human fibroblasts during *in vitro* ageing (Shevitz *et al,* 1986). Taking advantage of the peculiar behaviour of fibroblasts in relation to the age of the embryo, we have investigated in the present study the rate of fibronectin synthesis and its expression at the cell surface during adhesion of 8d and 16d CEF.

MATERIALS AND METHODS

Cell cultures

Fibroblasts were obtained from 8 d (8d CEF) and 16 d (16d CEF) old chick embryos using the method previously described by Aubery and Bourrillon (1976). Cells from primary monolayer cultures were used before confluence and were obtained by trypsin-TPCK treatment as previously reported (Botti *et al,* 1987).

Adhesion assays

Cell adhesion assays were conducted as previously described in 24 well tissue culture plates (Linbro, Poly Labo Paul Block and Cie, Strasbourg) coated or not with FN (20 µg/ml of human FN) in the absence of serum (Botti *et al,* 1987). The plates were incubated at 37 °C for various periods of time. At the end of the incubation period, the plates were washed 3 times with PBS to remove the non-adherent cells. The number of adherent cells was determined after a mild trypsin treatment. In some sets of experiments, the synthesis of endogenous attachment factors was prevented by incubating the cells with 25 µg/ml of cycloheximide for 2 h. The inhibitor was present at the same concentration in all the medias subsequently used.

Binding of iodinated FN

FN was iodinated as previously described (Codogno *et al,* 1987). 1 x 10^7 trypsin-treated cells/ml were suspended in Dulbecco's modified MEM, 20 mmol/l HEPES, 2% bovine serumalbumin pH 7.4. ^{125}I-FN at concentrations ranging from 0.1–10 nmol/l was added to 100 µl of cell suspension. The binding mixtures were then incubated for 2 h at 22 °C. The non-specific binding was determined in the presence of a 100-fold excess of unlabelled FN. Results were analysed according to Scatchard (1949).

Biosynthesis of FN

Eight d and 16d CEF were labelled with [^{14}C-U]-leucine (10 µCi/ml, sp act 300 Ci/mmol CEA, Saclay) for 30, 60 and 180 min. The newly synthesized FN was immunoprecipitated from intact cells using anti-human plasma fibronectin antibodies, (Dakopatts Sebia, Issy-les-Moulineaux) according to the method of Tarone *et al* (1980) and analyzed by polyacrylamide gel electrophoresis. In order to follow the kinetics of FN biosynthesis, 8 and 16d CEF were labelled with ^{35}S-methionine (10 µCi/ml, sp act 1200 Ci/mmol,

CEA, Saclay) for various times from 15–180 min. Radiolabelled FN was then immunoprecipitated from cell lysate.

Endo H treatment of FN

Cells were pulsed for 10 min with ^{35}S-methionine as described above and chased from 0–24 h in the presence of 2 mmol/l methionine. The labelled intracellular FN was immunoprecipitated and aliquots were either untreated (control) or treated with endo-β-N-acetylglucosaminidase H (endo H) according to the method of Tarentino and Maley (1974). SDS-polyacrylamide gel electrophoresis and autoradiographic analysis were conducted according to Laemmli (1970) and Bonner and Laskey (1974) respectively.

RESULTS

Cell adhesion

When plated on a plastic support, 8d CEF adhere more rapidly than 16d CEF, since 50% of adhesion was obtained in 110 min and 150 min respectively. Complete re-adhesion (80% of the plated cells) needed 4 h and 6 h in 8 and 16d CEF respectively. Whatever the age of embryos, the cells were unable to attach in the presence of cycloheximide. When plated on a FN-coated substrate, 8d and 16d CEF exhibit identical adhesion kinetics. The adhesion process was almost complete after 60 min for both fibroblast populations *ie* 80% of the cells were attached. The same results were observed in the absence or in the presence of cycloheximide.

FN binding sites on 8-d and 16-d CEF

Each cell population displays only 1 class of FN-binding sites and the same number of binding sites (550 000 sites/cell) with a k_d of 1.40 μmol/l.

Re-expression of FN at the cell surface

The ^{14}C-leucine-labelled FN was detected from 30 min in 8d CEF and the amount of FN increased up to 3 h of re-adhesion assay. In 16d CEF, the labelled FN was detected only after 60 min. Thus, the newly synthesized FN was expressed earlier at the cell surface in 8d than in 16d CEF (fig 1).

Kinetics of FN biosynthesis

The rate of ^{35}S-methionine incorporation was identical in 8d and 16d CEF from 0–60 min. Thereafter, a plateau was observed in 8d CEF, and this may represent the equilibrium between newly synthesized FN and secreted FN. In contrast, such a plateau was not observed in 16d CEF, in rela-

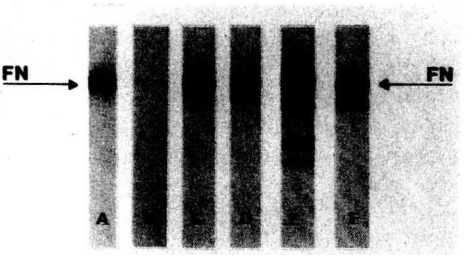

Fig 1. Expression of membrane fibronectin at various times of re-adhesion assay in 8d and 16d CEF. ^{14}C-leucine-labelled fibronectin was immunoprecipitated at various times with anti-human plasma fibronectin. SDS–polyacrylamide gel electrophoresis was performed on 7.5% acrylamide gels and analysed by autoradiography: 30 min, A) 8-d, B) 16-d; 60 min; C) 8-d, D) 16-d; 180 min, E) 8-d, F) 16-d.
At 180 min: other bands represent degradation products of fibronectin.

tion with the later expression of FN at the cell surface (fig 2).

Endo H sensitivity of FN

FN was shown to possess at least 6 sites of N-glycosylation substituted with complex type chain, synthesized in mannose-rich precursor form sensitive to endo H treatment (Olden *et al,* 1980). FN originated from 8d CEF was sensitive to endo H treatment until 20 min, and then became unsensitive. In 16d CEF, the FN exhibited an endo H susceptibility until 60 min after labelling with ^{35}S-methionine (table I). These results suggest that the time of efficient maturation of this secreted glycoprotein from the rough endoplasmic reticulum to the Golgi complex is 3-fold higher in 16d than in 8d CEF.

DISCUSSION

Fibroblasts obtained from 8 d and 16 d old chick embryos exhibited marked differences in their adhesive capacities on plastic support, whereas both 8d and 16d CEF display identical adhesion properties on FN-coated substratum. Unequal adhesion on plastic cannot be due to a difference in the expression of membrane FN

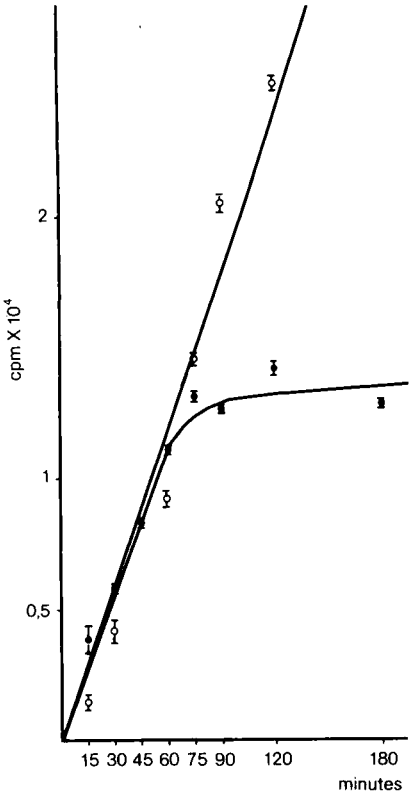

Fig 2. Kinetics of fibronectin biosynthesis in 8d and 16d CEF. After metabolic labelling with ^{35}S-methionine, fibronectin was immunoprecipitated from the cell lysate and the radioactivity was determined using a liquid scintillation spectrometer intertechnique SL 4000. ●: 8d CEF. ○: 16d CEF.

Table I. Fibronectin susceptibility to endo H treatment in 8d and 16d CEF.

	Chase time						
	0 min	*10 min*	*20 min*	*30 min*	*40 min*	*60 min*	*24 h*
8d	+	+	+	−	−	−	−
16d	+	+	+	+	+	+	−

After ^{35}S-methionine incorporation for 10 min in 8d and 16d CEF, the labelled fibronectin was immunoprecipitated at various times from 0–24 h of chase in the presence of 2mmol/l methionine. Aliquots of immunoprecipitates were treated with endo H. Untreated and treated immunoprecipitates are subjected to SDS-5% polyacrylamide gel electrophoresis followed by autoradiography. The susceptibility to endo H treatment was estimated from the modification of FN molecular weight.

receptors since 8d and 16d CEF expressed an identical number of FN receptors with similar affinity. In addition, FN interacts with high molecular weight membrane proteins (150–125 kDa) unsensitive to trypsin treatment in both 8d and 16d CEF (Botti et al, 1987). A difference in the FN expression at the cell surface is the most likely explanation. This hypothesis was supported by the results reported here since we observed that FN reappeared more rapidly in 8d than in 16d CEF. This difference in the FN re-expression at the cell surface is not due to a difference in the biosynthesis rate of the polypeptide chain. Like glycoproteins destined to secretion, FN is synthesized in the rough endoplasmic reticulum (RER) where asparagine linked mannose-rich built up on dolichol are transferred to the polypeptide backbone and trimmed into a complex chain along their passage in the Golgi apparatus. Preliminary studies showed that the transfer en bloc of oligosaccharide chain to the polypeptide occurred at almost the same rate in 8d and 16d CEF (data not shown). Thereafter, the delayed sensitivity to endo H of 16d FN was due to steps occurring during the transit between the RER and median Golgi. Moreover, differences in the late maturation of complex oligosaccharides in the trans Golgi could not be excluded. Nevertheless, no important variations in complex type oligosaccharides of FN between 8d and 16d CEF were observed (data not shown). From the data presented here we can conclude that early differences in the post-translational modification of N-glycans influence the CEF adhesion.

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